Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for Titration of Pseudorabies Virus in Vaccines

Date: October 29, 1998

Supersedes: SAM 118, Dated: March 1, 1989

Number: MVSAM0118.01

Standard Requirement: 9 CFR 113.318

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1. Introduction

This is an *in vitro* test method which utilizes viral plaque forming units (PFU) in a cell culture system to titer pseudorabies virus (PRV) in modified-live vaccines.

2. Materials

2.1 Equipment/instrumentation

2.1.1 36° ± 2°C, 5% ± 1% CO_2, high humidity incubator[1] meeting the requirements of the current version of GDOCSOP004

2.1.2 Vortex mixer[2]

2.1.3 Blender[3]

2.1.4 Micropipettors: 200 µl and 1000 µl single channel[4]

2.1.6 Water bath[5]

2.1.7 Self-refilling, 2-ml repetitive syringe[6]

2.2 Reagents/supplies

2.2.1 PRV Reference Virus,[7] Shope strain

2.2.2 Madin-Darby bovine kidney[8] (MDBK) cells

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[1] Model 3158, Forma Scientific Inc., Box 649, Marietta, OH 45750-0649 or equivalent

[2] Vortex-2 Genie, Model G-560, Scientific Industries Inc., 70 Orville Dr., Bohemia, NY 11716 or equivalent

[3] Waring blender, Cat. No. 14-509-35, Fisher Scientific Inc., 319 W. Ontario, Chicago, IL 60610 or equivalent

[4] Pipetman, Rainin Instrument Co., Mack Rd., Box 4026, Woburn, MA 01888 or equivalent

[5] Cat. No. 15-461-10, Fisher Scientific Inc. or equivalent

[6] Wheaton, Cat. No. 13-689-50C, Fisher Scientific Inc. or equivalent

[7] Reference quantities are available upon request from the Center for Veterinary Biologics-Laboratory (CVB-L), P.O. Box 844, Ames, IA 50010 or equivalent

[8] Cat. No. ATCC CCL-22, American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD 20852-1776

2.2.3 Minimum Essential Medium (MEM)

2.2.3.1 9.61 g MEM[9]

2.2.3.2 2.2 g sodium bicarbonate[10] ($NaHCO_3$)

2.2.3.3 Q.S. to 1000 ml with deionized water (DW), adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).[11]

2.2.3.4 Sterilize through 0.22-µm filter.[12]

2.2.3.5 Aseptically add:

1. 10 ml L-glutamine[13]

2. 5 ml lactalbumin hydrolysate or edamin[14]

3. 100 units/ml penicillin[15]

4. 50 µg/ml gentamicin sulfate[16]

5. 100 µg/ml streptomycin[17]

2.2.3.6 Store at 4° ± 2°C.

2.2.4 Growth Medium

2.2.4.1 900 ml of MEM

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[9] MEM with Earle's salts without sodium bicarbonate, Cat. No. 410-1500EF, Life Technologies, Inc., 8400 Helgerman Ct., Gaithersburg, MD 20884 or equivalent

[10] Cat. No. S 5761, Sigma Chemical, Inc., P.O. Box 14508, St. Louis, MO 63178 or equivalent

[11] Cat. No. 9535-01, J.T. Baker, Inc., 222 Red School Ln., Phillipsburg, NJ 08865 or equivalent

[12] Cat. No. 12122, Gelman Sciences, 600 S. Wagner Rd., Ann Arbor, MI 48106 or equivalent

[13] L-glutamine-200 mM (100X), liquid, Cat. No. 320-503PE, Life Technologies, Inc. or equivalent

[14] Edamin S, Cat. No. 59102, Sheffield Products, P.O. Box 630, Norwick, NY 13815 or equivalent

[15] Cat. No. 0049-0530-28, Schering Laboratories, 2000-T Galloping Hill Rd., Kenilworth, NJ 07033 or equivalent

[16] Cat. No. 0061-0464-04, Schering Laboratories or equivalent

[17] Cat. No. S-9137, Sigma Chemical, Inc. or equivalent

2.2.4.2 Aseptically add 100 ml fetal bovine serum (FBS), heat-inactivated at 56° ± 2°C for 30 ± 5 min.

2.2.4.3 Store at 4° ± 2°C.

2.2.5 2X Medium

2.2.5.1 100 ml 10X MEM[18]

2.2.5.2 2.2 g sodium bicarbonate

2.2.5.3 340 ml DW

2.2.5.4 Sterilize through 0.22-µm filter.

2.2.5.5 Aseptically add:

1. 100 units/ml penicillin

2. 50 µg/ml gentamicin sulfate

3. 100 µg/ml streptomycin

4. 50 ml FBS

2.2.5.6 Store at 4° ± 2°C.

2.2.6 2% Tragacanth Gum (Trag)

2.2.6.1 20 g Trag[19]

2.2.6.2 1000 ml DW

2.2.6.3 Mix vigorously small amounts at a time with a blender set on high.

2.2.6.4 Pour 500 ml each into 1000-ml media bottles.

2.2.6.5 Sterilize by autoclaving at 15 psi for 30 min.

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[18] MEM with Earle's salts 10X liquid, Cat. No. 410-11430, Life Technologies, Inc. or equivalent
[19] Acros AC42138-5000, Fisher Scientific, Inc. or equivalent

2.2.6.6 Store at 4° ± 2°C.

2.2.7 Overlay Medium

2.2.7.1 Mix equal volumes of 2X Medium and 2% Trag.

2.2.7.2 Store at 4° ± 2°C.

2.2.8 70% Ethyl Alcohol

2.2.8.1 74 ml ethyl alcohol[20]

2.2.8.2 26 ml DW

2.2.8.3 Store at room temperature (RT) (23° ± 2°C).

2.2.9 Crystal Violet Stain

2.2.9.1 7.5 g crystal violet[21]

2.2.9.2 50 ml 70% ethyl alcohol

2.2.9.3 Dissolve crystal violet in alcohol.

2.2.9.4 250 ml formaldehyde[22]

2.2.9.5 Q.S. to 1000 ml with DW, filter through filter paper.[23]

2.2.9.6 Store at RT.

2.2.10 Tissue culture plates, 6 well[24]

2.2.11 12x75-mm polystyrene tubes[25]

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[20] Denatured, 190 proof, Cat. No. 7018, J.T. Baker, Inc. or equivalent

[21] Cat. No. C0775, Sigma Chemical Co. or equivalent
[22] Cat. No. F79, Fisher Scientific, Inc. or equivalent

[23] Whatman #1, Cat. No. 1001, Fisher Scientific, Inc. or equivalent

[24] Falcon 3046, Becton Dickinson Labware, Becton Dickinson & Co., 2 Oak Park, Bedford, MA 01730 or equivalent

[25] Falcon 2058, Becton Dickinson Labware or equivalent

2.2.12 10-ml syringes[26] and needles[27]

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel must have training in the immunologic basis of virus titration assays, cell culture maintenance, and in the principles of aseptic techniques.

3.2 Preparation of equipment/instrumentation

Set the water bath at 36° ± 2°C.

3.3 Preparation of reagents/controls

3.3.1. Two days prior to test performance

Seed 6-well tissue culture plates with MDBK cells, in Growth Medium, at a cell count that will produce a monolayer after 48 ± 6 hr of incubation at 36° ± 2°C. These become the MDBK plates. Growth Medium is changed if excess acidity of the medium is observed as indicated by a change from red to yellow of Growth Medium.

3.3.2 On day of test performance

Rapidly thaw a vial of PRV Reference Virus in a water bath at 36° ± 2°C. Dilute the virus in 4.5 ml MEM to contain 15-40 PFU/100 µl.

3.4 Preparation of the sample (on day of test performance)

3.4.1 Rehydrate a vial of the Test Vaccine according to the manufacturer's instructions with a 10-ml syringe and 20-gauge needle. Allow to sit for 15 ± 5 min at RT.

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[26] Luer-Lok®, Cat. No. 309604, Becton Dickinson Labware or equivalent

[27] 20 gauge, 1.5 in, Cat. No. 250107, Becton Dickinson Labware or equivalent

3.4.2 Prepare serial 10-fold dilutions of test vaccine. Serial 10-fold dilutions may be made as follows:

3.4.2.1 Place 4.5 ml of MEM with the repetitive syringe into labeled 12x75-mm polystrene tubes.

3.4.2.2 Pipet 500 µl of test vaccine to the 10^{-1} tube, mix by vortexing. Discard pipet tip.

3.4.2.3 Repeat step **3.4.2.2** to the remaining tubes. Continue as needed (10^{-2} to 10^{-5}), transferring 500 µl from previous tube to the next dilution.

4. Performance of the test

4.1 Decant the Growth Media from MDBK plates.

4.2 Pipet 100 µl/well from each dilution of test vaccine to 2 wells of a MDBK plate. Mix by gentle swirling.

4.3 Pipet 100 µl/well of the diluted Reference Virus Control to 2 wells of 1 MDBK plate. Mix by gentle swirling.

4.4 Maintain 2 or more wells as uninoculated cell culture controls.

4.5 Incubate inoculated plates at 36° ± 2°C in a CO_2 atmosphere for 60 ± 10 min for virus adsorption.

4.6 Add 3.0 ml/well of Overlay Medium (see section **2.2.8**) to the plates. Discard any unused, warmed Overlay Medium.

4.7 Incubate the MDBK plates undisturbed at 36° ± 2°C in a CO_2 atmosphere for 96 ± 12 hr.

4.8 At the end of incubation, decant Overlay Medium. Pipet 2 ml of the Crystal Violet Stain (see section **2.2.10**) into each well of the plates using the repetitive syringe.

4.9 Allow plates to stand at RT for 15 ± 5 min.

4.10 Discard the Crystal Violet Stain down a sink. Wash the cell monolayers by dipping each plate several times in a container of running water from the cold faucet. Allow to air dry.

4.11 PFU counting

4.11.1 The PFU are visible as clear, circular areas in the cell monolayer where the cells have been destroyed by the virus.

4.11.2 Count the number of PFU for each well.

4.11.2.1 Average the number of PFU between the duplicate wells for each test vaccine dilution.

4.11.2.2 Average the number of PFU between the 2 wells of the Reference Virus Control wells.

4.11.3 Determine the virus titers and express as PFU/dose. Take the dilution of a test vaccine that contains an average of at least 30 PFU's. Example:

Log_{10} of plaque count (30)	1.48
Log_{10} of dilution counted (10^{-3})	3.00
Log_{10} of 2-ml dose factor (20)	1.30
Virus titer/dose (total)	5.78

The test vaccine contains $10^{5.78}$ PFU/2-ml dose.

5. Interpretation of the test results

5.1 The test is invalid if visible contamination is observed in all dilutions of a Test Vaccine.

5.2 For a valid assay, the Reference Virus Control must have an average PFU count between 15-40.

5.3 Any test not meeting the criteria of **5.1** and **5.2** is considered a NO TEST and may be repeated without prejudice.

5.4 One plaque represents a single infective unit whereas the 50% endpoint infective dose (one ID_{50}) is statistically equivalent to a theoretical 0.69 of an infective unit. Fifty percent endpoints will be 1.44 times those expressed as PFU/unit of inoculation. Therefore, to express PFU titer as 50% tissue culture infective dose ($TCID_{50}$) titer, multiply the PFU by 1.44 or add 0.16 to the log_{10} value of the PFU titer. In the example above, the $TCID_{50}$ would be 5.78 + 0.16 = 5.94 or $10^{5.94}$ $TCID_{50}$/2-ml dose.

5.5 If the validity requirements are not met, then the assay is considered a NO TEST and can be retested without prejudice.

5.6 If the validity requirements are met and the titer of the vaccine is greater than or equal to the titer contained in the filed outline of production for the product under test, the product is considered satisfactory.

5.7 If the validity requirements are met but the titer of the Test Vaccine is lower than the required minimum, it must be retested according to 9 CFR 113.8.

6. Report of test results

6.1 Test results are reported as the virus titer in $TCID_{50}$/dose.

6.2 Record all test results on the test record.

7. References

7.1 Conrath TB.: Handbook of Microtiter Procedures. 1972. Clinical and Research Applications Laboratory, Cooke Engineering Company, Alexandria, VA.

8. Changes

8.1 This document was rewritten to meet the current NVSL/CVB QA requirements, to clarify practices currently in use in the CVB-L, and to provide additional detail. No significant changes were made from the previous protocol.